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The impact of short chain fatty acids on GLP-1 and PYY secretion from the isolated perfused rat colon

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Abstract

The colonic epithelium harbors a large number of endocrine cells, but little is known about the endocrine functions of the colon. However, the high density of GLP-1 and PYY secreting L-cells is of great interest because of the potential anti-diabetic and anti-obesity effects of GLP-1 and PYY. Short chain fatty acids (SCFAs) produced by local bacterial fermentation are suggested to activate the colonic free fatty acid receptors FFAR2 (GPR43) and FFAR3 (GPR41), stimulating the colonic L-cells. We used the isolated perfused rat colon as a model of colonic endocrine secretion and studied the effects of the predominant SCFAs formed: acetate, propionate and butyrate. We show that luminal and especially vascular infusion of acetate and butyrate significantly increases colonic GLP-1 secretion, and to a minor extent also PYY secretion, but only after enhancement of intracellular cAMP. Propionate neither affected GLP-1 nor PYY secretion whether administered luminally or vascularly. A FFAR2 and FFAR3 specific agonist (CFMB/AR420626) had no effect on colonic GLP-1 output, and a FFAR3 antagonist (AR399519) did not decrease the SCFA-induced GLP-1 response. However, the voltage-gated Ca^{2+}-channel blocker nifedipine, the K_{ATP}-channel opener diazoxide and the ATP synthesis inhibitor 2,4-dinitrophenol completely abolished the responses. FFAR2 receptor studies confirmed low-potent partial agonism of acetate, propionate and butyrate, compared to CFMB which is a full agonist with around 750-fold higher potency than the SCFAs. In conclusion, SCFAs may increase colonic GLP-1/PYY secretion, but FFAR2/FFAR3 do not seem to be involved. Rather, SCFAs are metabolized and appear to function as a colonocyte energy source.

New & Noteworthy

By the use of *in situ* isolated perfused rat colon we show that SCFAs primarily are used as a colonocyte energy source in the rat, subsequently triggering GLP-1 secretion *independent* of FFAR2 and FFAR3. Opposite many previous studies on SCFAs, FFAR2/FFAR3 and GLP-1 secretion, this experimental model allows investigation of the physiological interactions between luminal nutrients and secretion from cells whose function depend critically on their blood supply as well as nerve and paracrine interactions.

Introduction

The colon serves to store and transport indigestible material, to absorb water and salt from the ileal chyme, and is also home for fermentation processes converting undigested and malabsorbed substances into absorbable moieties such as short chain fatty acids (SCFAs) (4, 28). However, the colonic mucosa also harbors a large number of enteroendocrine cells, the density of which increases towards the distal part of the gut (20, 23, 63). Very little is known about the endocrine secretion from the colon and about its endocrine functions in general, but the high abundance of the glucagon like peptide-1 (GLP-1) and peptide-YY (PYY) secreting L-cells has attracted considerable interest because of the potential anti-diabetic and anti-obesity effects of GLP-1 and PYY (30). In fact, analogs of GLP-1 are now widely used in the treatment of type 2 diabetes (T2D) (1, 19).

In 1995 Deacon et al. demonstrated that the human colon produces biologically active GLP-1 ($7\text{-}36_{NH2}$) in amounts that are large enough to have physiological importance (17). However, the best characterized stimulants for GLP-1 secretion *in vivo* are nutrients including glucose, amino acids and long chain fatty acids, which are absorbed in the proximal small intestine and do not reach the distal colonic L-cells (22, 38, 53, 60). Accordingly, colectomized individuals have normal GLP-1 meal responses (51). Interestingly, however, L-cell secretion has been found to be increased in individuals with short-bowel syndrome but preserved colon (31). The latter suggests that the colonic cells are able to produce a significant amount of proglucagon-derived peptides, including GLP-1, which may reach the general circulation. The potential importance of this may be illustrated by patients undergoing gastric bypass surgery, who exhibit markedly increased postprandial secretion of GLP-1 and PYY, which seems to be involved in the diabetes remission and at least part of the weight loss resulting from the operation (29). Stimulation of the endogenous secretion of these hormones may therefore have important therapeutic potential, but to accomplish this goal, a better understanding of the mechanisms that regulate their secretion from the colon is necessary.

Recently, changes in the gut microbiota have been linked to a variety of disorders such as T2D and obesity. It is not clear exactly how the microbiota influences host metabolism, but a proposed mechanism involves the generation of SCFAs (11, 28). The amount of SCFAs ingested with foods is minor, but a considerable production of SCFAs takes place in the distal gut as a result of bacterial fermentation of non-absorbed carbohydrate, often in the form of dietary fibers that resist digestion and absorption in the small intestine (15, 16). The primary SCFAs (>95%) generated from intestinal fermentation are acetate (C2), propionate (C3), and butyrate (C4), some of which may be absorbed and delivered to the systemic circulation (15, 32, 42). Circulating plasma levels of SCFAs are in the lower micromolar range, whereas luminal concentrations of SCFAs in the colon may reach 100 mM (5, 18, 57, 69).

SCFAs have been proposed as an energy source, as enhancer of intestinal growth and as signal molecules in colonic motility (12) and ion transport (33, 36). Moreover, they are hypothesized to constitute the link between the gut microbiota and distal L-cell activity, as certain cell studies have reported increased secretion of GLP-1 and/or PYY upon SCFA administration (7, 26, 41, 48, 58, 68, 70). The mechanism by which acetate, propionate and butyrate exert their actions is believed to involve GPCRs, namely FFAR2 (GPR43) and FFAR3 (GPR41) (8, 40). Both FFAR2 and FFAR3 are reported to be expressed on L-cells in the colon, whereas expression in the small intestine is more debated (33, 48, 68). FFAR3 is found to signal exclusively through the $G\alpha_i$-coupled pathway, which typically is linked to suppression of gut hormone secretion. FFAR2 is likewise $G\alpha_i$-coupled, but may also activate $G\alpha_q$-signaling (8, 59, 68), making FFAR2 a more likely candidate for stimulating L-cell secretion. Whether FFAR2 and FFAR3 reside on the basolateral or apical membranes of the L-cells and whether they primarily detect luminal or vascular SCFAs remains to be explored.

The aim of the current study was to investigate the presumed positive effects of SCFAs on colonic GLP-1 and PYY secretion, including an evaluation of FFAR2 and FFAR3 as possible significant targets for the treatment of metabolic disorders. In order to do so, we used the isolated perfused rat colon as a model of colonic endocrine secretion, because it maintains correct cell polarity and vascular integrity as opposed to isolated cell cultures. Also, any observed effect can solely be allocated to the colon as the confounding influence of the rest of the body, inherent in *in vivo* studies, is avoided by isolating the organ.

Materials and Methods

Animals

Handling of the donor animals was performed in accordance with international accepted guidelines and with permission from the Danish Animal Experiments Inspectorate (license no. 2013-15-2934-00833). Male Wistar rats (Janvier, Saint Berthevin Cedex, France) fed ad libitum and weighing between 250-300 g were used as donors. Animals were housed two per cage under a 12:12 hour light-dark cycle. After approximately one week of acclimatization they were used for experiments. The rats were anaesthetized with a subcutaneous injection of Hypnorm/Midazolam (0.0158 mg fentanyl citrate + 0.5 mg fluanisone + 0.25 mg midazolam / 100 g) before surgery.

Isolation of the colon

After lack of reflexes was established, the operation was started by a midline incision exposing the abdominal cavity. The colon was cut immediately after the cecum and again approximately 10 cm distal

from cecum (average length: 10.20±0.84 cm), so that the most distal colon and rectum were not included in the preparation. Next, all vessels supplying the small intestine, the cecum and the spleen were ligated, thus avoiding perfusion of these segments. The stomach was removed after ligating the esophagus and tying off all blood supplies. Following removal of the stomach, the renal kidney stalks were ligated, and the celiac artery partly perfusing the pancreas was ligated too. Tubing was inserted into the proximal colonic lumen to establish a route for luminal stimulation, and the colonic contents were washed out by gentle flushing with pre-warmed saline. The distal lumen was left open allowing contents to exit. Subsequently, aorta was ligated proximally to the superior mesenteric artery and immediately afterwards a metal catheter (1.0 mm diameter) was placed into the distal aorta and fixed with a ligature and vascular perfusion was started. Next, a draining metal catheter (1.3 mm diameter) was placed into the vena portae. Finally, the rat was euthanized and the colon kept artificially alive.

Experimental protocol

After isolation, the colon was perfused *in situ* at a constant vascular flow rate of 3 ml/min while saline was continuously infused into the colonic lumen (0.05 ml/min). After a 30 min equilibrium period each protocol started with a 10 min baseline period followed by addition of test substances. Vascularly administered test substances were infused for 10 min at a flow rate of 0.15 ml/min, while luminally administered stimulations were infused for 20 min. Luminal test substances were infused at an initial rate of 0.25 ml/min for the first 5 min (to replace the saline solution in the lumen) and then at 0.15 ml/min throughout the rest of the stimulation period. Following luminal stimulations saline was luminally infused at 0.25 ml/min for 5 min to remove test substances. The total venous effluent was collected for 1 min periods and stored at -20°C until analysis. We used equipment dedicated for rodent organ perfusion (Hugo Sachs Elektronik, March-Hugstetten, Germany). The perfusion buffer was a modified Krebs-Ringer bicarbonate buffer, containing in addition 5% dextran T-70 (Pharmacosmos, Denmark, cat.no. 40014), 0.1% bovine serum albumin (Merck, cat.no. 1.12018.0500), 3.5 mM glucose and 5 mM pyruvate, fumarate and glutamate. Perfusion buffer was heated to 37°C and continuously gassed throughout the experiment with 95% O_2 and 5% CO_2 to achieve pH 7.4 and a high oxygen partial pressure. Respiration (calculated from the partial pressures of CO_2 of arterial and venous perfusion buffer samples; CO_2 excretion: median=17.8 µl/g, IQR=8.5-23.6 µl/g), stable perfusion pressure and vascular effluent flow rate were monitored throughout the experiments and used as an indication of the organ's wellbeing.

Test substances

Bombesin (positive control, Bachem, cat.no. H-2155) was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, cat.no. 67-68-5) and perfusion buffer and added to give a final perfusate concentration of 10 nM. 3-

Isobutyl-1-methylxanthine (IBMX, Sigma Aldrich, cat.no. I5879) was dissolved in DMSO and further diluted in perfusion buffer resulting in a final perfusate concentration of 10 μM. Stock solutions of acetate (Sigma Aldrich, cat.no. 695092), propionate (Sigma Aldrich, cat.no. 81910) and butyrate (Sigma Aldrich, cat.no. B-103500) were diluted in H_2O and the pH value was adjusted to 7.4 by addition of 5 M NaOH. For vascular infusions (at 1 mM) the solutions were further diluted in perfusion buffer while they were further diluted in saline when added to the lumen (100 mM). AR420626 and AR399519 (kind gifts from Thue Schwartz, NNF CBMR, Department of Biomedical Sciences, University of Copenhagen, Denmark) and CFMB ((S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-yl)butamide, Merck Millipore, cat.no. 371725) were dissolved in DMSO and diluted in perfusion buffer to reach final concentrations of 10 μM. Nifedipine (Sigma Aldrich, cat.no N7634), diazoxide (Sigma Aldrich, cat.no D9035) and 2,4-dinitrophenol (Sigma Aldrich, cat.no D198501) were also dissolved in DMSO and diluted in perfusion buffer to final perfusate concentrations of 10-250 μM. Final concentrations of DMSO never exceeded 1% which did not influence secretion when given alone (see below).

Hormone analysis

GLP-1 and PYY concentrations in the venous effluent were analyzed using radioimmunoassays (RIA). GLP-1 concentrations were determined using an in-house antiserum, codename 89390, which is specific for the C-terminal part of amidated GLP-1 isoforms (7-36_{NH2} and 9-36_{NH2}) (50). We chose to measure amidated GLP-1 rather than glycine-extended GLP-1 as the amidated forms dominate in rats (37, 65). Synthetic GLP-1 7-36_{NH2} (Bachem, cat.no H-6795.0500) was used as standard and ^{125}I-labeled GLP-1 7-36_{NH2} (a gift from Novo Nordisk A/S, Bagsværd, Denmark) as tracer. Total PYY immunoreactivity was measured with a porcine antiserum (Bachem, cat.no T-4093) using synthetic rat/porcine PYY (Bachem, cat.no H-6042) as standard and ^{125}I-labeled porcine PYY (Perkin Elmer Life Sciences, cat.no NEX240) as tracer (65).

Mass spectrometry

For analysis of SCFAs we used the method of (27) with minor modifications. In brief, 40 μL of the venous effluent or perfusion buffer were mixed with 10 μL 200 mM 3-nitrophenylhydrazine (3-NPH) in 50% ethanol and 120 μL N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and 6% pyridine in 50% ethanol and incubated at room temperature for 30 min while shaking. This and all subsequent procedures were run in polypropylene plastic tubes or well plates in order to avoid SCFA contamination from glassware and several other plastics. The mixtures were diluted to 1 ml with 10% ethanol and 100 μL internal standard (IS) was added. IS was prepared by derivatizing 50 μL of a solution of 20 mM acetic acid, 10 mM propionic acid and 5 mM butyric acid and 1 mg $^{13}C_6$-3NPH hydrochloride in 50% ethanol with 25 μL 120 mM EDC and 25 μL 6% pyridine in 50% ethanol and mixing at room temperature for 30 min. A dilution series of external standards

196 prepared of all three SCFAs from 10-1250 μM and an assay blank (50% ethanol) were all treated as
197 described for the effluent samples. Samples, sample pools and all standards and assay blanks (all 10 μL)
198 were injected in random order into a Waters (Milford, CA) Acquity UPLC coupled through an electrospray
199 interface to a triple quadrupole MS/MS system and concentrations determined using the vendor software
200 (Quanlynx). The BEH C18 1.7 μm, 2.1 x 5 mm column was eluted at 40°C by a gradient going from 20%-
201 100% acetonitrile with 0.01% formic acid. All MS-MS transitions, dwell times and collision energies were as
202 described in (27). The CV% for pools (n=5) in the analytical batch was <10% and r^2 for the external standard
203 calibration curve was >0.99.
204
205 *Transfection and Tissue Culture*
206 COS-7 cells were cultured at 10% CO_2 and 37°C in Dulbecco's modified Eagle's medium 1885 supplemented
207 with 10% foetal bovine serum, 2 mM glutamine, 180 units/ml penicillin, and 45 g/ml streptomycin.
208 Transient transfection of COS-7 cells with human and rat FFAR2 for the IP_3 assays was performed using the
209 calcium phosphate precipitation method with the addition of chloroquine (35).
210
211 *IP_3 assay*
212 One day after transfection, COS-7 cells transiently expressing either human or rat FFAR2 (35,000 cells/well)
213 were incubated with *myo*-[^{3}H]inositol (5 μl/ml, 2 μCi/ml) in 0.1 ml of medium overnight in a 96-well plate.
214 The following day, cells were washed twice in PBS and incubated in 0.1 ml of Hanks' balanced salt solution
215 (Invitrogen) supplemented with 10 mM LiCl at 37°C in the presence of various concentrations of acetate,
216 propionate, butyrate, and FFAR2 agonist (CFMB) for 90 min. Assay medium was then removed, and cells
217 were extracted by the addition of 50 μl of 10 mM formic acid to each well, followed by incubation on ice for
218 30–60 min. The [^{3}H]inositol phosphates in the formic acid cell lysates were thereafter quantified by adding
219 yttrium silicate-poly-D-Lys-coated SPA beads (66). Briefly, 35 μl of cell extract was mixed with 80 μl of SPA
220 bead suspension in H_2O (12.5 μg/μl) in a white 96-well plate. Plates were sealed and shaken on table shaker
221 for at least 30 min. SPA beads were allowed to settle and react with the extract for at least 8 hours before
222 radioactivity was determined using a Packard Top Count NXT^{TM} scintillation counter (PerkinElmer Life
223 Sciences).
224
225 *Statistical analysis*
226 Hormone secretion was calculated as hormone output (effluent concentration x perfusion flow). Statistical
227 analysis of colonic hormone responses was performed by comparing mean basal outputs prior to infusion
228 with mean outputs during infusion (5 min periods for vascular stimulations, 10 min periods for luminal

stimulations, all starting 2 min after infusion start and also stated in the figure legends). For two identical consecutive responses, statistical analysis of hormone secretion was performed by comparing the mean output during the two responses using a paired t-test (except for figure 2B and 2D and except for the comparison of vascular vs. luminal responses where an unpaired t-test was used). All statistics were performed using GraphPad Prism 6. Values of $P<0.05$ were considered significant and all data in the text and graphs are presented as mean±SEM.

Results

Acetate and butyrate, but not propionate, increase colonic GLP-1 and PYY secretion in the presence of IBMX

Vascular (1mM) or luminal (100mM) stimulation with acetate, propionate or butyrate had no impact on GLP-1 or PYY output from the perfused rat colon in the absence of the phosphodiesterase inhibitor IBMX, despite greatly enhanced secretion in response to intra-arterial stimulation with 10 nM of our positive control bombesin (figure 1). Supplementing the perfusion buffer with 10 µM IBMX throughout the experiments led to a significant increase in GLP-1 responses to vascular acetate and butyrate administration and similar responses were obtained after repeated stimulation (figure 2A and E. P-values for repeated stimulation were GLP-1: acetate; p=0.278, butyrate; p=0.199, PYY: acetate; p=0.517, butyrate; p=0.443). PYY secretion was also significantly elevated upon both 1 mM acetate and butyrate administration, however, to a lesser extent than GLP-1 secretion (figure 2A and E). Luminal administration of 100 mM acetate led to a short-lasting peak in GLP-1 secretion whereas PYY secretion remained unaffected (figure 2B). Luminal infusion of 100 mM butyrate significantly increased GLP-1 and PYY secretion from the isolated perfused rat colon (figure 2F). Propionate had no impact on colonic GLP-1 or PYY secretion whether administered vascularly or luminally (figure 2C and D). In all experiments, intra-arterial administration of 10 nM bombesin was included as a positive control at the end, and did in each case result in a robust GLP-1 and PYY response (figure 2A-F).

Vascular co-administration of a cocktail solution of acetate and butyrate significantly increased GLP-1 and PYY secretion from the perfused rat colon (figure 3A). Moreover, co-administration of acetate/butyrate showed a dose-dependent GLP-1 and PYY response, as colonic GLP-1 output increased 1.74-fold and colonic PYY output increased 1.60-fold when comparing responses to 0.1 mM with responses to 1 mM (figure 3A). In addition, co-administration had an additive effect on the colonic GLP-1 response compared to infusion of acetate or butyrate alone (figure 3B). PYY output was, however, not significantly changed when compared to a single SCFA infusion (figure 3B). Furthermore, colonic GLP-1 and PYY output increased upon luminal infusion of 100 mM acetate/butyrate (figure 3C). However, the response to luminal co-administration of acetate/butyrate seemed fairly similar to luminal butyrate infusion alone, in accordance with data showing

that luminal acetate alone only has minor impact on colonic hormone secretion (figure 3D). Comparing the mean output during vascular infused acetate/butyrate (1 mM) to the mean output during luminal acetate/butyrate (100 mM) infusion demonstrated that vascular stimulation resulted in a significantly higher GLP-1 response compared to the response induced by luminal stimulation (vascular: 67.6±7.3 fmol/min vs. luminal: 37.0±2.9 fmol/min, p=0.0097). PYY responses to vascular and luminal acetate/butyrate administration were not significantly different (vascular: 28.9±2.9 fmol/min vs. luminal: 26.7±2.5 fmol/min, p=0.755).

We also measured absorption of acetate, propionate and butyrate to the vascular circulation after luminal infusion (figure 4). The concentrations reached in the venous effluent were comparable to those applied vascularly (~1 mM). However, also prestimulatory vascular acetate levels were high; this turned out to be due to presence of acetate in our perfusion buffer (perfusion buffer content: acetate; 909 µM, propionate; 73 µM, butyrate; below detection limit (65 µM)).

FFAR2 and FFAR3 activation have no impact on GLP-1 secretion, while a FFAR3 agonist increases PYY release from the perfused rat colon

Next, we investigated the importance of the GPCRs, free fatty acid receptor-2 and -3 (FFAR2 and FFAR3), for the SCFA-induced colonic GLP-1 and PYY response. 10 µM FFAR2 specific agonist, CFMB, had no significant impact on GLP-1 and PYY output from the perfused rat colon, possibly indicating that FFAR2 is not necessarily involved in colonic hormone secretion (figure 5A). The FFAR3 specific agonist, AR420626, did not change colonic GLP-1 output, but 10 µM AR420626 significantly elevated PYY secretion (figure 5C). However, a FFAR3 specific antagonist, AR399519, did not decrease the GLP-1 or the PYY response induced by a cocktail infusion of 1 mM acetate and butyrate, suggesting that the acetate/butyrate mediated hormone response is independent of FFAR3 signaling (figure 5D). Control experiments evaluating the impact of intra-arterial DMSO administration (1% solution similar to the amount of DMSO used to dissolve test substances), revealed that DMSO alone had no impact on GLP-1 or PYY output from the perfused rat colon (GLP-1; 17.1±0.6 to 19.8±1.2 fmol/min, PYY; 15.9±1.2 to 16.8±0.6 fmol/min, n=2, data not shown).

The FFAR3 specific agonist and antagonist have previously been shown to selectively activate and inhibit the $G\alpha_i$-coupling of FFAR3 (21). FFAR2 couples to $G\alpha_i$ as well as to $G\alpha_q$, and to our knowledge the FFAR2 specific agonist CFMB and the SCFAs have not been tested in terms of their abilities to induce FFAR2-mediated $G\alpha_q$ activity. An IP3 assay was therefore conducted on transiently transfected COS-7 cells expressing either human or rat FFAR2. Data showed low-potent, weak partial agonism of acetate, propionate, and butyrate on both human and rat FFAR2. For the human FFAR2 the EC_{50} and E_{max} values were 0.5 mM and 64%, 0.3 mM and 69%, and 0.5 mM and 42% for acetate, propionate and butyrate, respectively, compared to CFMB (figure 5B and table 1). For rat FFAR2 the EC_{50} value was 0.17 mM for all

three SCFAs, however, their E_{max} values ranged from 81%, 93% and 57% for acetate, propionate and butyrate, respectively, compared to CFMB (figure 5B and table 1). CFMB on the other hand appeared to be a stronger agonist both on human and rat FFAR2 with an approximately 350-600-fold better potency than the SCFAs on the human FFAR2 and a 750-fold better potency than the SCFAs on the rat FFAR2 (EC_{50} of 0.8 and 0.2 µM on human and rat FFAR2, respectively) (figure 5B and table 1). This is consistent with the potency of CFMB identified in $G\alpha_i$-mediated FFAR2 activity (21), demonstrating that lack of colonic hormone response to CFMB was not due to lack of activation of FFAR2 if present.

SCFAs stimulate colonic GLP-1 and PYY secretion trough depolarization and Ca^{2+}-influx

Finally, we investigated the role of the voltage-gated Ca^{2+}-channel blocker nifedipine, the K_{ATP}-channel opener diazoxide and the ATP synthesis inhibitor 2,4-dinitrophenol for the SCFA-induced colonic GLP-1 and PYY response. Blockage of voltage-gated Ca^{2+}-channels (by 10 µM nifedipine) significantly reduced the GLP-1 and PYY responses, suggesting that Ca^{2+}-influx resulting from depolarization is involved in the SCFA-mediated hormone secretion (figure 6A and B). The importance of depolarization was validated by administration of 250 µM diazoxide, which completely abolished the SCFA-induced GLP-1 and PYY response as well as basal hormone secretion (figure 6C and D). Furthermore, inhibiting intracellular ATP synthesis by 10 µM 2,4-dinitrophenol also prevented acetate/butyrate mediated GLP-1 and PYY secretion (figure 6E and F) while responses to bombesin were unaffected by preceding 2,4-dinitriphenol.

Discussion

Following the therapeutic success of incretin-based anti-diabetic drugs, research has focused on the L-cell asking whether it would be possible and beneficial to stimulate the endogenous release of GLP-1 *in vivo.* Moreover, the dramatic increases in GLP-1 and PYY secretion seen after bariatric surgery seem to arise from distal sources, potentially involving colonic L-cells (29, 43). Understanding the mechanisms underlying GLP-1 release is essential for further progress. *In vivo,* L-cells are integrated within the epithelial cell layer and are therefore differentially exposed to luminal and plasma constituents at their apical and basolateral membrane surfaces (9). This environment almost certainly impacts L-cell function, but is impossible to reestablish in single cell studies. However, in the present study we made use of a robust procedure for isolation and perfusion of the rat colon, allowing physiologically relevant studies of the dynamics of colonic endocrine secretion. In combination with single cells, where *direct* effects on the L-cell can be demonstrated the full picture may then be assembled. As already alluded to, it is unclear to what extent the colon contributes to postprandial circulating GLP-1 and which stimuli are the most appropriate for colonic secretion. Also, the mechanisms underlying colonic GLP-1 and PYY release are incompletely understood. In the small intestine, GLP-1/PYY secretion depends on absorption and/or signaling properties

of digested nutrients (22, 38). However, since only limited amounts of digestible nutrients normally reach the colon, other stimuli for GLP-1 secretion, like SCFAs, might be involved.

First of all, our study demonstrated that the introduction of SCFAs had no effect on secretion from the rat colon unless the tissue was primed with a phosphodiesterase inhibitor (IBMX). This suggests that the SCFA signal is weak and needs interaction and probably potentiation with a cyclic nucleotide mechanism, most likely involving cAMP formation, which is known from studies of single colonic L-cells to provide a powerful stimulus to GLP-1 secretion (60, 68). Interestingly, unlike acetate and butyrate, propionate was without effect, comparable with previous work on perfused rat colon (55, 56). Secondly, our study demonstrated that vascular acetate and butyrate were stronger stimuli for GLP-1 secretion than luminal stimulation. Normally, circulating plasma levels of SCFAs are found in the lower micromolar range (acetate may reach 200 µM) (18, 57, 69), so the vascularly applied concentrations used in this study may be considered rather high. However, local concentrations of SCFA at the basolateral L-cell membrane in the colon may be significantly higher than circulating levels, considering that the absorbed SCFAs must reach a high concentration in the interstitial space before they enter and mix into the total vascular outflow from the gut. Among SCFAs, acetate is the one that reaches the highest concentrations both luminally and vascularly (44, 69), while butyrate appears to be the SCFA with lowest concentration in the general circulation, perhaps because butyrate is a preferred metabolic substrate of the colonocytes (5, 39, 57, 61). The lack of impact by both vascularly and luminally administered propionate was rather surprising, since we had no prior evidence to suggest that propionate would act differently from acetate and butyrate. Here we show that acetate, propionate and butyrate all cross the epithelial cell layer in the colon as indicated by the clear and relevant increases in vascular SCFA concentration upon luminal SCFA administration. Undissociated SCFAs may be absorbed by passive diffusion, while dissociated SCFAs (the primary form in the colonic lumen and in the present experiments) are absorbed by transporters present in the apical membrane (18). Acetate measurements revealed that our perfusion buffer, and therefore also baseline samples, contained a rather high amount of acetate, which appears to be a common problem derived from the numerous contacts with various glass utensils that are used in modern laboratories. This means that the basal colonic GLP-1 and PYY secretion is under a constant influence of acetate stimulation in our model, and that vascular acetate administration (1 mM) actually results in an increase in effluent acetate concentration from around 1 mM to 2 mM instead of 0 to 1 mM. Nevertheless, the applied dose did lead to an increased GLP-1 and PYY response, demonstrating that acetate is capable of influencing colonic endocrine secretion, but indicating that the acetate mediated hormone response observed from the perfused rat colon may have been greater if no acetate was already present.

The linking of GLP-1 release to FFAR3 stimulation has raised some interesting physiological questions as FFAR3 is found to signal exclusively through the $G\alpha_i$-pathway (8), which traditionally is linked to suppressed secretion (59). FFAR2 is likewise $G\alpha_i$-coupled, but also couples to $G\alpha_q$-signaling, activating phospholipase C and thereby triggering IP_3 mediated Ca^{2+} release and secretion of peptide hormones. Therefore, FFAR2 activation is suggested to dominate over any FFAR3 signaling induced by SCFAs with regards to increased hormone release (6, 8, 68). Nevertheless, we observed increased secretion of PYY when the rat colon was vascularly stimulated with the FFAR3 specific agonist, AR420626, supporting that FFAR3 is expressed in the murine colon (48, 68), but only adding to the confusion regarding the stimulation mechanism. GLP-1 secretion, however, was not affected by AR420626 suggesting that different signaling pathways may be responsible for colonic GLP-1 and PYY secretion and/or that the peptides are not co-secreted but arise from different L-cells with different expression profiles as also suggested previously for small intestinal L-cells (65). Moreover, Nøhr et al. showed enhanced GLP-1 secretion (PYY not measured) from murine colonic crypt cultures in response to the same selective FFAR2 ($G\alpha_i/G\alpha_q$) and FFAR3 ($G\alpha_i$) agonists applied in our study (48). Similarly, Park et al. found increased GLP-1 secretion from human NCH-H716 cells in response to a selective inverse agonist for FFAR2 (BTI-A-404), while 10 mM propionate had no effect (52). A third FFAR2 ligand, AZ1729, was without effect on GLP-1 secretion from mouse colonic crypts, though it was found to be a potent activator of FFAR2 $G\alpha_i$-signaling (6). In the isolated perfused rat colon, however, the FFAR2 selective agonist and the FFAR3 selective antagonist had no impact on colonic hormone output. This was observed despite the FFAR3 antagonist, AR399519, previously has been demonstrated to selectively inhibit FFAR3 in stably transfected HEK293 cells (21). We tested both human and rat FFAR2, and found that the FFAR2 specific ligand, CFMB, is a full agonist to human as well as rat FFAR2. Hence, the negative results here seem to exclude an important action of FFAR2 on L-cell secretion in the rat colon. In our lab we have studied the effects of both the FFAR2 and the FFAR3 selective agonists using the isolated perfused mouse pancreas, and both showed significant potentiation of somatostatin secretion (Ørgaard et al., unpublished), demonstrating that the compounds are compatible with our experimental approach. Together, the data confirm that the ligands applied were administered in an active form and that missing impact on colonic hormone release was not due to trivial technical problems. Furthermore, in the receptor studies, SCFAs as well as CFMB activated FFAR2-mediated $G\alpha_q$ activity; however, acetate, propionate and butyrate all showed low-potent, partial weak agonism towards human as well as rat FFAR2, indicating that the possible interaction of SCFAs with FFAR2 is not physiologically relevant. Among the SCFAs, propionate was found to be the most potent ligand on rat FFAR2, but as already mentioned propionate was found to have no effect on colonic endocrine secretion. Thus, our data suggest that neither FFAR2 nor FFAR3 are necessarily involved in the enhanced colonic hormone secretion mediated by SCFAs in the rat. Other, less investigated,

receptor candidates for SCFAs include the rat olfactory receptor Olr59 (mouse ortholog Olfr78, human ortholog OR51E2) which is shown to induce $G\alpha_s$-mediated cAMP accumulation upon activation (45). The receptor is widely expressed in the sensory neurons, while Olfr78/GLP-1 co-expression is found to be very limited (16%) (24). GPR109A represents yet another possible SCFA target, and seems to be involved in regulation of inflammation (14). The receptor is activated by high concentrations of butyrate (1 mM), but not by acetate and propionate (2, 34). Moreover, activation of GPR109A induces $G\alpha_i$-mediated intracellular cAMP inhibition (2, 64). Olr59 and GPR109A activation are therefore unlikely to explain the results of the present study.

Studies on L-cell secretion have mainly focused on nutrient-induced small intestinal hormone release. Glucose is the most extensively studied secretagogue and consensus seems to be that uptake initiates membrane depolarization, and perhaps closure of ATP-sensitive K^+ channels, which cause a rise in intracellular Ca^{2+}, triggering the Ca^{2+}-dependent exocytosis of GLP-1 containing vesicles (3, 38, 53, 60). Opening of voltage-gated Ca^{2+}-channels seems also to be involved in the SCFA-mediated GLP-1 and PYY response, as inhibition of these channels by nifedipine administration completely blocked the responses. The K_{ATP}-channel opener diazoxide causes hyperpolarization of the L-cells as opening of these channels leads to further efflux of K^+. When co-infused with the acetate and butyrate cocktail, diazoxide caused complete attenuation of the colonic hormone response to these SCFAs, and also inhibited the basal secretion, indicating that the actual membrane potential and the ion currents behind it are essential for the colonic hormone secretion. Moreover, SCFA-induced GLP-1 and PYY secretion were lost by blockage of ATP synthesis by 2,4-dinitrophenol indicating that SCFAs may be metabolized by the colonic cells and act via stimulation of ATP production and possibly actions on the K_{ATP}-channels (either in the L-cell or in a putative stimulating neighbour cell) as suggested above. Interestingly, the secretory response to the $G\alpha_q$-activator bombesin (our positive control), which stimulates GLP-1 secretion by phospholipase C activation and mobilization of intracellular Ca^{2+}, rather than by depolarization, was not affected by the prior 2,4-dinitrophenol infusion, supporting that this additional mechanism for colonic hormone secretion remained unaffected by the acute ATP-depletion. Taken together, data lead us to suggest that acetate and butyrate are taken up by the L-cells followed by intracellular metabolism, which impacts the ATP/ADP ratio end causes L-cell membrane depolarization leading to GLP-1 and PYY secretion by activation of voltage-gated Ca^{2+}-channels and uptake of extracellular Ca^{2+}. This would be consistent with the inability of propionate to stimulate secretion since this 3-carbon molecule is poorly metabolized in the peripheral tissues of most mammals (13, 61).

Through secretion of GLP-1 and PYY, SCFAs may indirectly affect host metabolism by increasing satiety and decreasing gastric emptying and gut motility (25, 47). Additionally, SCFA-mediated GLP-1 secretion may exert a beneficial effect on glucose metabolism by potentiating glucose-stimulated insulin secretion from pancreatic beta-cells (46). The most important observations regarding the relationship between SCFAs and L-cell secretion derive from studies of the ultimate model: the living human being. However, the mechanistic approach applied in our study is not possible in humans, and therefore we find the perfused rat colon the best model for our study, though caution obviously is needed when translating findings to humans. Currently, evidence regarding an influence of SCFAs on hormone secretion in humans is rather sparse, but SCFAs have been reported to affect PYY, but not GLP-1, secretion when rectally infused into the human colonic lumen (10, 70). In contrast, colonic SCFA administration to healthy humans has been found to have no effect on plasma PYY or GLP-1 although H_2 in exhaled air (signal of colonic fermentation) increased (62). This is again contrasted by a study where colonic fermentation, stimulated by one week's fructo-oligosaccharide intake, increased plasma GLP-1, but not PYY, in patients with gastroesophageal reflux disease (54). Short bowel patients with intact colon show increased fasting plasma GLP-1 and PYY levels (31) as well as increased responses to an oral carbohydrate intake (49), indicating that the endocrine secretion from the colon may be of physiological importance. A relationship between high fibre intake and satiety has been found in other human studies (16), but whether this is a direct effect of SCFA production and GLP-1 secretion is not known. Taken together, SCFAs may be able to impact PYY and/or GLP-1 secretion in humans, but due to the complex nature of clinical studies and conflicting results, more research is needed in order clarify the role of SCFAs. Since L-cells are numerous in the colon and the colon is able to produce active GLP-1 (17, 23, 63), the endocrine colon has the potential to influence our overall metabolic status. Normally, however, the effects of GLP-1 derived from the colon may largely be local such as delaying transit time as seen in the small intestine (67). This could also be a result of large amounts of SCFAs generated from bacterial fermentation. However, looking at the present study, data suggest that in the rat acetate and butyrate are primarily used as a colonocyte energy source, being metabolized intracellularly to ATP. This may subsequently lead to L-cell depolarization activating voltage-gated Ca^{2+}-channels and triggering the Ca^{2+}-dependent exocytosis of peptide containing vesicles, all of it independent of FFAR2 and FFAR3. Further studies are required to understand endocrine functions and the therapeutic potential of the colonic endocrine cells.

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References

1. Agerso H, Jensen LB, Elbrond B, Rolan P, and Zdravkovic M. The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy men. *Diabetologia* 45: 195-202, 2002.

2. Ahmed K, Tunaru S, and Offermanns S. GPR109A, GPR109B and GPR81, a family of hydroxy-carboxylic acid receptors. *Trends in pharmacological sciences* 30: 557-562, 2009.

3. Albrechtsen NJW, Kuhre RE, Deacon CF, and Holst JJ. Targeting the intestinal L-cell for obesity and type 2 diabetes treatment. *Expert Review of Endocrinology & Metabolism* 9: 61-72, 2014.

4. Bharucha AE. Lower gastrointestinal functions. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society* 20 Suppl 1: 103-113, 2008.

5. Bloemen JG, Venema K, van de Poll MC, Olde Damink SW, Buurman WA, and Dejong CH. Short chain fatty acids exchange across the gut and liver in humans measured at surgery. *Clinical Nutrition* 28: 657-661, 2009.

6. Bolognini D, Moss CE, Nilsson K, Petersson AU, Donnelly I, Sergeev E, Konig GM, Kostenis E, Kurowska-Stolarska M, Miller A, Dekker N, Tobin AB, and Milligan G. A Novel Allosteric Activator of Free Fatty Acid 2 Receptor Displays Unique Gi-functional Bias. *The Journal of biological chemistry* 291: 18915-18931, 2016.

7. Brooks L, Viardot A, Tsakmaki A, Stolarczyk E, Howard JK, Cani PD, Everard A, Sleeth ML, Psichas A, Anastasovskaj J, Bell JD, Bell-Anderson K, Mackay CR, Ghatei MA, Bloom SR, Frost G, and Bewick GA. Fermentable carbohydrate stimulates FFAR2-dependent colonic PYY cell expansion to increase satiety. *Molecular metabolism* 6: 48-60, 2017.

8. Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM, Wise A, and Dowell SJ. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *The Journal of biological chemistry* 278: 11312-11319, 2003.

9. Buffa R, Capella C, Fontana P, Usellini L, and Solcia E. Types of endocrine cells in the human colon and rectum. *Cell and tissue research* 192: 227-240, 1978.

10. Canfora EE, van der Beek CM, Jocken JWE, Goossens GH, Holst JJ, Olde Damink SWM, Lenaerts K, Dejong CHC, and Blaak EE. Colonic infusions of short-chain fatty acid mixtures promote energy metabolism in overweight/obese men: a randomized crossover trial. *Scientific reports* 7: 2360, 2017.

11. Chakraborti CK. New-found link between microbiota and obesity. *World journal of gastrointestinal pathophysiology* 6: 110-119, 2015.

12. **Cherbut C, Ferrier L, Roze C, Anini Y, Blottiere H, Lecannu G, and Galmiche JP. Short-chain fatty acids modify colonic motility through nerves and polypeptide YY release in the rat. *The American journal of physiology* 275: G1415-1422, 1998.**
13. **Clausen MR, and Mortensen PB. Kinetic studies on colonocyte metabolism of short chain fatty acids and glucose in ulcerative colitis. *Gut* 37: 684-689, 1995.**
14. **Correa-Oliveira R, Fachi JL, Vieira A, Sato FT, and Vinolo MA. Regulation of immune cell function by short-chain fatty acids. *Clinical & translational immunology* 5: e73, 2016.**
15. **Cummings JH, Pomare EW, Branch WJ, Naylor CP, and Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 28: 1221-1227, 1987.**
16. **Darzi J, Frost GS, and Robertson MD. Do SCFA have a role in appetite regulation? *The Proceedings of the Nutrition Society* 70: 119-128, 2011.**
17. **Deacon CF, Johnsen AH, and Holst JJ. Human colon produces fully processed glucagon-like peptide-1 (7-36) amide. *FEBS letters* 372: 269-272, 1995.**
18. **den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, and Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of lipid research* 54: 2325-2340, 2013.**
19. **Edwards CM, Stanley SA, Davis R, Brynes AE, Frost GS, Seal LJ, Ghatei MA, and Bloom SR. Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers. *American journal of physiology Endocrinology and metabolism* 281: E155-161, 2001.**
20. **Eissele R, Goke R, Willemer S, Harthus HP, Vermeer H, Arnold R, and Goke B. Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. *European journal of clinical investigation* 22: 283-291, 1992.**
21. **Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nohr MK, Pan J, Sinz CJ, Carrington PE, Akiyama TE, Jones RM, Tang C, Ahmed K, Offermanns S, Egerod KL, Zigman JM, and Schwartz TW. Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. *Molecular metabolism* 2: 376-392, 2013.**
22. **Ezcurra M, Reimann F, Gribble FM, and Emery E. Molecular mechanisms of incretin hormone secretion. *Current opinion in pharmacology* 13: 922-927, 2013.**
23. **Ferri GL, Adrian TE, Ghatei MA, O'Shaughnessy DJ, Probert L, Lee YC, Buchan AM, Polak JM, and Bloom SR. Tissue localization and relative distribution of regulatory peptides in separated layers from the human bowel. *Gastroenterology* 84: 777-786, 1983.**
24. **Fleischer J, Bumbalo R, Bautze V, Strotmann J, and Breer H. Expression of odorant receptor Olfr78 in enteroendocrine cells of the colon. *Cell and tissue research* 361: 697-710, 2015.**
25. **Flint A, Raben A, Astrup A, and Holst JJ. Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. *The Journal of clinical investigation* 101: 515-520, 1998.**
26. **Freeland KR, Wilson C, and Wolever TM. Adaptation of colonic fermentation and glucagon-like peptide-1 secretion with increased wheat fibre intake for 1 year in hyperinsulinaemic human subjects. *The British journal of nutrition* 103: 82-90, 2010.**
27. **Han J, Lin K, Sequeira C, and Borchers CH. An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography-tandem mass spectrometry. *Analytica chimica acta* 854: 86-94, 2015.**
28. **Hartstra AV, Bouter KE, Backhed F, and Nieuwdorp M. Insights into the role of the microbiome in obesity and type 2 diabetes. *Diabetes care* 38: 159-165, 2015.**
29. **Holst JJ. Enteroendocrine secretion of gut hormones in diabetes, obesity and after bariatric surgery. *Current opinion in pharmacology* 13: 983-988, 2013.**
30. **Holst JJ. The physiology of glucagon-like peptide 1. *Physiological reviews* 87: 1409-1439, 2007.**
31. **Jeppesen PB, Hartmann B, Thulesen J, Hansen BS, Holst JJ, Poulsen SS, and Mortensen PB. Elevated plasma glucagon-like peptide 1 and 2 concentrations in ileum resected short bowel patients with a preserved colon. *Gut* 47: 370-376, 2000.**

32. Kaji I, Karaki S, and Kuwahara A. Short-chain fatty acid receptor and its contribution to glucagon-like peptide-1 release. *Digestion* 89: 31-36, 2014.
33. Karaki S, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, Furness JB, and Kuwahara A. Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. *Cell and tissue research* 324: 353-360, 2006.
34. Kasubuchi M, Hasegawa S, Hiramatsu T, Ichimura A, and Kimura I. Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients* 7: 2839-2849, 2015.
35. Kissow H, Hartmann B, Holst JJ, Viby NE, Hansen LS, Rosenkilde MM, Hare KJ, and Poulsen SS. Glucagon-like peptide-1 (GLP-1) receptor agonism or DPP-4 inhibition does not accelerate neoplasia in carcinogen treated mice. *Regulatory peptides* 179: 91-100, 2012.
36. Kripke SA, Fox AD, Berman JM, Settle RG, and Rombeau JL. Stimulation of Intestinal Mucosal Growth with Intracolonic Infusion of Short-Chain Fatty Acids. *Journal of Parenteral and Enteral Nutrition* 13: 109-116, 1989.
37. Kuhre RE, Albrechtsen NW, Windelov JA, Svendsen B, Hartmann B, and Holst JJ. GLP-1 amidation efficiency along the length of the intestine in mice, rats and pigs and in GLP-1 secreting cell lines. *Peptides* 55: 52-57, 2014.
38. Kuhre RE, Frost CR, Svendsen B, and Holst JJ. Molecular mechanisms of glucose-stimulated GLP-1 secretion from perfused rat small intestine. *Diabetes* 2014.
39. Layden BT, Angueira AR, Brodsky M, Durai V, and Lowe WL, Jr. Short chain fatty acids and their receptors: new metabolic targets. *Translational research : the journal of laboratory and clinical medicine* 161: 131-140, 2013.
40. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, Parmentier M, and Detheux M. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *The Journal of biological chemistry* 278: 25481-25489, 2003.
41. Lin HV, Frassetto A, Kowalik EJ, Jr., Nawrocki AR, Lu MM, Kosinski JR, Hubert JA, Szeto D, Yao X, Forrest G, and Marsh DJ. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. *PloS one* 7: e35240, 2012.
42. Macfarlane S, and Macfarlane GT. Regulation of short-chain fatty acid production. *The Proceedings of the Nutrition Society* 62: 67-72, 2003.
43. Madsbad S, Dirksen C, and Holst JJ. Mechanisms of changes in glucose metabolism and bodyweight after bariatric surgery. *The lancet Diabetes & endocrinology* 2: 152-164, 2014.
44. Mascord D, Smith J, Starmer GA, and Whitfield JB. Effects of increasing the rate of alcohol metabolism on plasma acetate concentration. *Alcohol and alcoholism (Oxford, Oxfordshire)* 27: 25-28, 1992.
45. Mosienko V, Chang AJ, Alenina N, Teschemacher AG, and Kasparov S. Rodents and humans are able to detect the odour of L-Lactate. *PloS one* 12: e0178478, 2017.
46. Nauck MA, Homberger E, Siegel EG, Allen RC, Eaton RP, Ebert R, and Creutzfeldt W. Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses. *The Journal of clinical endocrinology and metabolism* 63: 492-498, 1986.
47. Nauck MA, Niedereichholz U, Ettler R, Holst JJ, Orskov C, Ritzel R, and Schmiegel WH. Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans. *The American journal of physiology* 273: E981-988, 1997.
48. Nohr MK, Pedersen MH, Gille A, Egerod KL, Engelstoft MS, Husted AS, Sichlau RM, Grunddal KV, Poulsen SS, Han S, Jones RM, Offermanns S, and Schwartz TW. GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. *Endocrinology* 154: 3552-3564, 2013.
49. Olesen M, Gudmand-Hoyer E, Holst JJ, and Jorgensen S. Importance of colonic bacterial fermentation in short bowel patients: small intestinal malabsorption of easily digestible carbohydrate. *Digestive diseases and sciences* 44: 1914-1923, 1999.

603 **50. Orskov C, Rabenhoj L, Wettergren A, Kofod H, and Holst JJ. Tissue and plasma**
604 **concentrations of amidated and glycine-extended glucagon-like peptide I in humans. *Diabetes* 43: 535-**
605 **539, 1994.**
606 **51. Palnaes Hansen C, Andreasen JJ, and Holst JJ. The release of gastric inhibitory peptide,**
607 **glucagon-like peptide-I, and insulin after oral glucose test in colectomized subjects. *Scandinavian journal***
608 ***of gastroenterology* 32: 473-477, 1997.**
609 **52. Park BO, Kim SH, Kong GY, Kim DH, Kwon MS, Lee SU, Kim MO, Cho S, Lee S, Lee HJ, Han**
610 **SB, Kwak YS, Lee SB, and Kim S. Selective novel inverse agonists for human GPR43 augment GLP-1**
611 **secretion. *European journal of pharmacology* 771: 1-9, 2016.**
612 **53. Parker HE, Reimann F, and Gribble FM. Molecular mechanisms underlying nutrient-**
613 **stimulated incretin secretion. *Expert reviews in molecular medicine* 12: e1, 2010.**
614 **54. Piche T, des Varannes SB, Sacher-Huvelin S, Holst JJ, Cuber JC, and Galmiche JP. Colonic**
615 **fermentation influences lower esophageal sphincter function in gastroesophageal reflux disease.**
616 ***Gastroenterology* 124: 894-902, 2003.**
617 **55. Plaisancie P, Dumoulin V, Chayvialle JA, and Cuber JC. Luminal glucagon-like peptide-1(7-**
618 **36) amide-releasing factors in the isolated vascularly perfused rat colon. *The Journal of endocrinology***
619 **145: 521-526, 1995.**
620 **56. Plaisancie P, Dumoulin V, Chayvialle JA, and Cuber JC. Luminal peptide YY-releasing factors**
621 **in the isolated vascularly perfused rat colon. *The Journal of endocrinology* 151: 421-429, 1996.**
622 **57. Pouteau E, Meirim I, Metairon S, and Fay LB. Acetate, propionate and butyrate in plasma:**
623 **determination of the concentration and isotopic enrichment by gas chromatography/mass spectrometry**
624 **with positive chemical ionization. *Journal of mass spectrometry : JMS* 36: 798-805, 2001.**
625 **58. Psichas A, Sleeth ML, Murphy KG, Brooks L, Bewick GA, Hanyaloglu AC, Ghatei MA, Bloom**
626 **SR, and Frost G. The short chain fatty acid propionate stimulates GLP-1 and PYY secretion via free fatty**
627 **acid receptor 2 in rodents. *International journal of obesity (2005)* 39: 424-429, 2015.**
628 **59. Reimann F, and Gribble FM. A tag to track short chain fatty acid sensors. *Endocrinology***
629 **154: 3492-3494, 2013.**
630 **60. Reimann F, Habib AM, Tolhurst G, Parker HE, Rogers GJ, and Gribble FM. Glucose sensing in**
631 **L cells: a primary cell study. *Cell metabolism* 8: 532-539, 2008.**
632 **61. Roediger WE. Utilization of nutrients by isolated epithelial cells of the rat colon.**
633 ***Gastroenterology* 83: 424-429, 1982.**
634 **62. Ropert A, Cherbut C, Roze C, Le Quellec A, Holst JJ, Fu-Cheng X, Bruley des Varannes S, and**
635 **Galmiche JP. Colonic fermentation and proximal gastric tone in humans. *Gastroenterology* 111: 289-296,**
636 **1996.**
637 **63. Sjolund K, Sanden G, Hakanson R, and Sundler F. Endocrine cells in human intestine: an**
638 **immunocytochemical study. *Gastroenterology* 85: 1120-1130, 1983.**
639 **64. Soga T, Kamohara M, Takasaki J, Matsumoto S, Saito T, Ohishi T, Hiyama H, Matsuo A,**
640 **Matsushime H, and Furuichi K. Molecular identification of nicotinic acid receptor. *Biochemical and***
641 ***biophysical research communications* 303: 364-369, 2003.**
642 **65. Svendsen B, Pedersen J, Jacob Wewer Albrechtsen N, Hartmann B, Torang S, Rehfeld JF,**
643 **Seier Poulsen S, and Holst JJ. An analysis of co-secretion and co-expression of gut hormones from male**
644 **rat proximal and distal small intestine. *Endocrinology* en20141710, 2014.**
645 **66. Thiele S, Steen A, Jensen PC, Mokrosinski J, Frimurer TM, and Rosenkilde MM. Allosteric**
646 **and orthosteric sites in CC chemokine receptor (CCR5), a chimeric receptor approach. *The Journal of***
647 ***biological chemistry* 286: 37543-37554, 2011.**
648 **67. Tolessa T, Gutniak M, Holst JJ, Efendic S, and Hellstrom PM. Inhibitory effect of glucagon-**
649 **like peptide-1 on small bowel motility. Fasting but not fed motility inhibited via nitric oxide**
650 **independently of insulin and somatostatin. *The Journal of clinical investigation* 102: 764-774, 1998.**

68. Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F, and Gribble FM. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. *Diabetes* 61: 364-371, 2012.

69. Topping DL, and Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological reviews* 81: 1031-1064, 2001.

70. van der Beek CM, Canfora EE, Lenaerts K, Troost FJ, Holst JJ, Masclee AAM, Dejong CHC, and Blaak EE. Colonic Acetate Infusions Promote Fat Oxidation and Improve Metabolic Parameters in Overweight Males. *Clinical Nutrition* 33: S16-S17.

Figure legends

Figure 1: GLP-1 and PYY secretion (fmol/min) from the isolated perfused rat colon in the absence of the phosphodiesterase inhibitor (IBMX), mean±SEM, n=4 in each group. A) 1 mM vascular acetate infusion (GLP-1: acetate 1^{st}; p=0.661, acetate 2^{nd}; p=0.643, PYY: acetate 1^{st}; p=0.391, acetate 2^{nd}; p=0.278). B) 100 mM luminal acetate infusion (GLP-1: p=0.898, PYY: p=0.078). C) 1 mM vascular propionate infusion (GLP-1: propionate 1^{st}; p=0.402, propionate 2^{nd}; p=0.324, PYY: propionate 1^{st}; p=0.592, propionate 2^{nd}; p=0.674). D) 100 mM luminal propionate infusion (GLP-1: p=0.406, PYY: p=0.260). E) 1 mM vascular butyrate infusion (GLP-1: butyrate 1^{st}; p=0.443, butyrate 2^{nd}; p=0.072, PYY: butyrate 1^{st}; p=0.092, butyrate 2^{nd}; p=0.237). F) 100 mM luminal butyrate infusion (GLP-1: p=0.249, PYY: p=0.540). A-F) 10 nM bombesin (vascularly) was included at the end of each experiment as a positive control.

Figure 2: GLP-1 and PYY secretion from the isolated perfused rat colon in the presence of 10 µM IBMX (fmol/min and baseline subtracted total output during 5 min (1^{st}: min 12-16, 2^{nd}: min 42-46) vascular (A,C,E) and 10 min (min 12-21) luminal (B,D,F) infusion), mean±SEM, n=6 in each group. * indicates significant increase from baseline (*p<0.05). A) 1 mM vascular acetate infusion (GLP-1: acetate 1^{st}; p=0.045, acetate 2^{nd}; p=0.029, PYY: acetate 1^{st}; p=0.029, acetate 2^{nd}; p=0.023). B) 100 mM luminal acetate infusion (GLP-1: p=0.021, PYY: p=0.951). C) 1 mM vascular propionate infusion (GLP-1: propionate 1^{st}; p=0.968, propionate 2^{nd}; p=0.444, PYY: propionate 1^{st}; p=0.067, propionate 2^{nd}; p=0.611). D) 100 mM luminal propionate infusion (GLP-1: p=0.468, PYY: p=0.328). E) 1 mM vascular butyrate infusion (GLP-1: butyrate 1^{st}; p=0.049, butyrate 2^{nd}; p=0.047, PYY: butyrate 1^{st}; p=0.027, butyrate 2^{nd}; p=0.022). F) 100 mM luminal butyrate infusion (GLP-1: p=0.012, PYY: p=0.002). A-F) 10 nM bombesin (vascularly) was included at the end of each experiment as a positive control.

Figure 3: GLP-1 and PYY secretion from the isolated perfused rat colon in the presence of IBMX, mean±SEM, n=6 in each group. * indicates significant increase from baseline (*p<0.05, **p<0.01, ***p<0.001) and Δ indicates significant difference between response values (Δp<0.05). A) Vascular infusion of a cocktail of 0.1 mM and 1 mM acetate/butyrate solution (fmol/min) (GLP-1: 0.1 mM cocktail; p=0.003, 1

mM cocktail; p=0.0005, PYY: 0.1 mM cocktail; p=0.003, 1 mM cocktail; p=0.005). C) Luminal infusion of 100 mM acetate/butyrate solution (fmol/min) (GLP-1: p=0.048, PYY: p=0.048). B) Baseline subtracted total GLP-1 and PYY output during 5 min (1^{st}: min 12-16, 2^{nd}: min 42-46) vascular infusions. Grey bars arise from figure 2 (GLP-1: from acetate alone 39.6±3.9 to cocktail 67.6±7.3 fmol/min; p=0.023, from butyrate alone 40.2±4.8 to cocktail 67.6±7.3 fmol/min; p=0.057, PYY: from acetate alone 25.0±1.7 to cocktail 28.9±2.9 fmol/min; p=0.413, from butyrate alone 24.4±2.1 to cocktail 28.9±2.9 fmol/min, p=0.408). D) Baseline subtracted total GLP-1 and PYY output during 10 min (min 12-21) luminal infusions. Grey bars arise from figure 2 (GLP-1: from acetate alone 22.5±1.0 to cocktail 34.0±2.5 fmol/min; p=0.008, from butyrate alone 33.6±1.9 to cocktail 34.0±2.5 fmol/min, p=0.850, PYY: from acetate alone 9.2±0.7 to cocktail 27.4±2.5 fmol/min; p=0.014, from butyrate alone 14.3±0.5 to cocktail 27.4±2.5 fmol/min, p=0.055). A/C) 10 nM bombesin was included at the end of each experiment as a positive control.

Figure 4: Measurements of acetate, propionate and butyrate in the effluent perfusion samples before and after SCFA administration (100 mM) to the lumen of the isolated perfused rat colon, mean±SEM, n=3-6 in each group. Ace: acetate, But: butyrate, Pro: propionate.

Figure 5: GLP-1 and PYY secretion from the isolated perfused rat colon in the presence of IBMX (fmol/min and baseline subtracted total output during 5 min (A: min 12-16, C: min 16-20, D: 1^{st}; min 12-16, 2^{nd}; min 52-56) vascular infusion), mean±SEM, n=6 in each group. * indicates significant increase from baseline (*p<0.05, **p<0.01). A) Vascular infusion of 10 µM FFAR2 agonist (CFMB) (GLP-1: p=0.088, PYY: p=0.150). B) IP_3 assay conducted on transient transfected COS-7 cells expressing either human or rat FFAR2. Acetate, propionate, and butyrate show low-potent partial weak agonism on both human and rat FFAR2 compared to CFMB, which is a full agonist towards FFAR2 C) Vascular infusion of 10 µM FFAR3 agonist (AR420626) (GLP-1: p=0.182, PYY: p=0.011). D) Vascular infusion of a 1 mM cocktail solution of acetate and butyrate followed by vascular administration of the same acetate/butyrate cocktail co-infused with 10 µM FFAR3 antagonist (AR399519) (GLP-1: cocktail 34.8±4.7 to cocktail +FFAR3 antagonist 35.6±3.7 fmol/min, p=0.922, PYY: cocktail 20.4±2.0 to cocktail +FFAR3 antagonist 25.8±2.1 fmol/min, p=0.142). A/C/D) 10 nM bombesin was included at the end of each experiment as a positive control.

Figure 6: GLP-1 and PYY secretion from the isolated perfused rat colon in the presence of IBMX, mean±SEM, n=6 in each group. * indicates significant increase from baseline (*p<0.05, **p<0.01, ***p<0.001) and Δ indicates significant difference between response values (Δp<0.05, ΔΔp<0.01). A) Vascular infusion of a 1 mM cocktail solution of acetate and butyrate followed by vascular administration of

same acetate/butyrate cocktail co-infused with 10 µM nifedipine (fmol/min) (GLP-1: cocktail 61.3±6.4 to cocktail +nifedipine 30.6±3.3 fmol/min, p=0.045, PYY: cocktail 40.1±2.5 to cocktail +nifedipine 24.1±1.9 fmol/min, p=0.043). C) Vascular infusion of a 1 mM cocktail solution of acetate and butyrate followed by vascular administration of same acetate/butyrate cocktail co-infused with 250 µM diazoxide (fmol/min) (GLP-1: cocktail 84.6±8.7 to cocktail +diazoxide 22.5±3.5 fmol/min, p=0.004, PYY: cocktail 42.7±2.9 to cocktail +diazoxide 13.6±0.9 fmol/min, p=0.003). E) Vascular infusion of a 1 mM cocktail solution of acetate and butyrate followed by vascular administration of same acetate/butyrate cocktail co-infused with 10 µM 2,4-dinitrophenol (fmol/min) (GLP-1: cocktail 56.6±6.5 to cocktail +2,4-dinitrophenol 24.2±24 fmol/min, p=0.007, PYY: cocktail 36.3±4.8 to cocktail +2,4-dinitrophenol 14.5±1.3 fmol/min, p=0.021). B/D/F) Baseline subtracted total GLP-1 and PYY output during 5 min (1^{st}: min 12-16, 2^{nd}: min 47-51) of infusion. A/C/E) 10 nM bombesin was included at the end of each experiment as a positive control.

Table 1: EC_{50} and E_{max} values for the FFAR2 agonist (CFMB) as well as for acetate, propionate, and butyrate on both human and rat FFAR2.

Figure 1

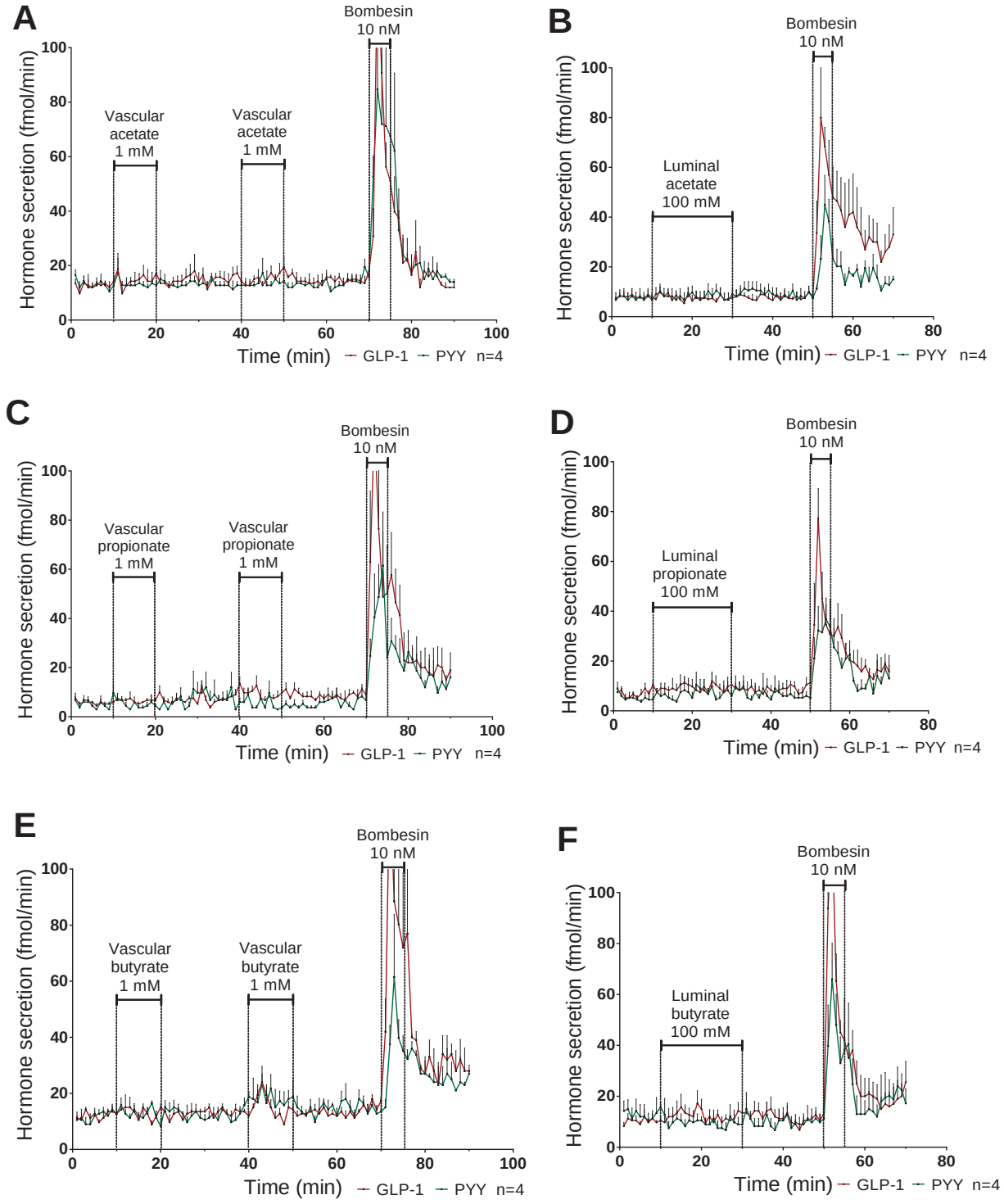
A
Bombesin
10 nM
Vascular
acetate
1 mM
Vascular
acetate
1 mM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=4
B
Bombesin
10 nM
Luminal
acetate
100 mM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=4
C
Bombesin
10 nM
Vascular
propionate
1 mM
Vascular
propionate
1 mM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=4
D
Bombesin
10 nM
Luminal
propionate
100 mM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=4
E
Bombesin
10 nM
Vascular
butyrate
1 mM
Vascular
butyrate
1 mM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=4
F
Bombesin
10 nM
Luminal
butyrate
100 mM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=4

Figure 2

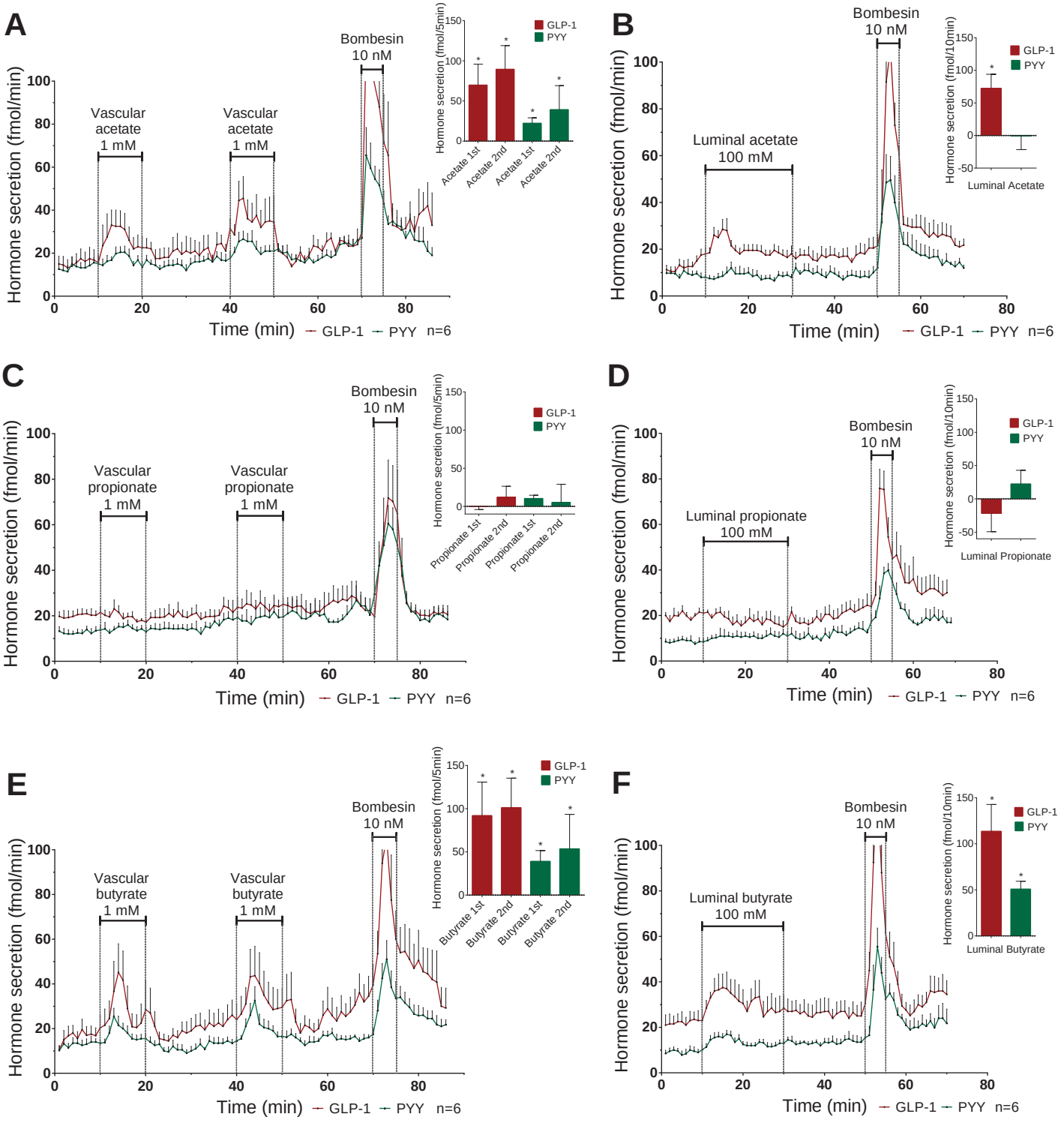
A
Vascular acetate 1 mM
Vascular acetate 1 mM
Bombesin 10 nM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=6
Hormone secretion (fmol/5min)
Acetate 1st
Acetate 2nd
Acetate 1st
Acetate 2nd
B
Luminal acetate 100 mM
Bombesin 10 nM
Hormone secretion (fmol/10min)
Luminal Acetate
C
Vascular propionate 1 mM
Vascular propionate 1 mM
Bombesin 10 nM
Propionate 1st
Propionate 2nd
Propionate 1st
Propionate 2nd
D
Luminal propionate 100 mM
Bombesin 10 nM
Luminal Propionate
E
Vascular butyrate 1 mM
Vascular butyrate 1 mM
Bombesin 10 nM
Butyrate 1st
Butyrate 2nd
Butyrate 1st
Butyrate 2nd
F
Luminal butyrate 100 mM
Bombesin 10 nM
Luminal Butyrate

Figure 3

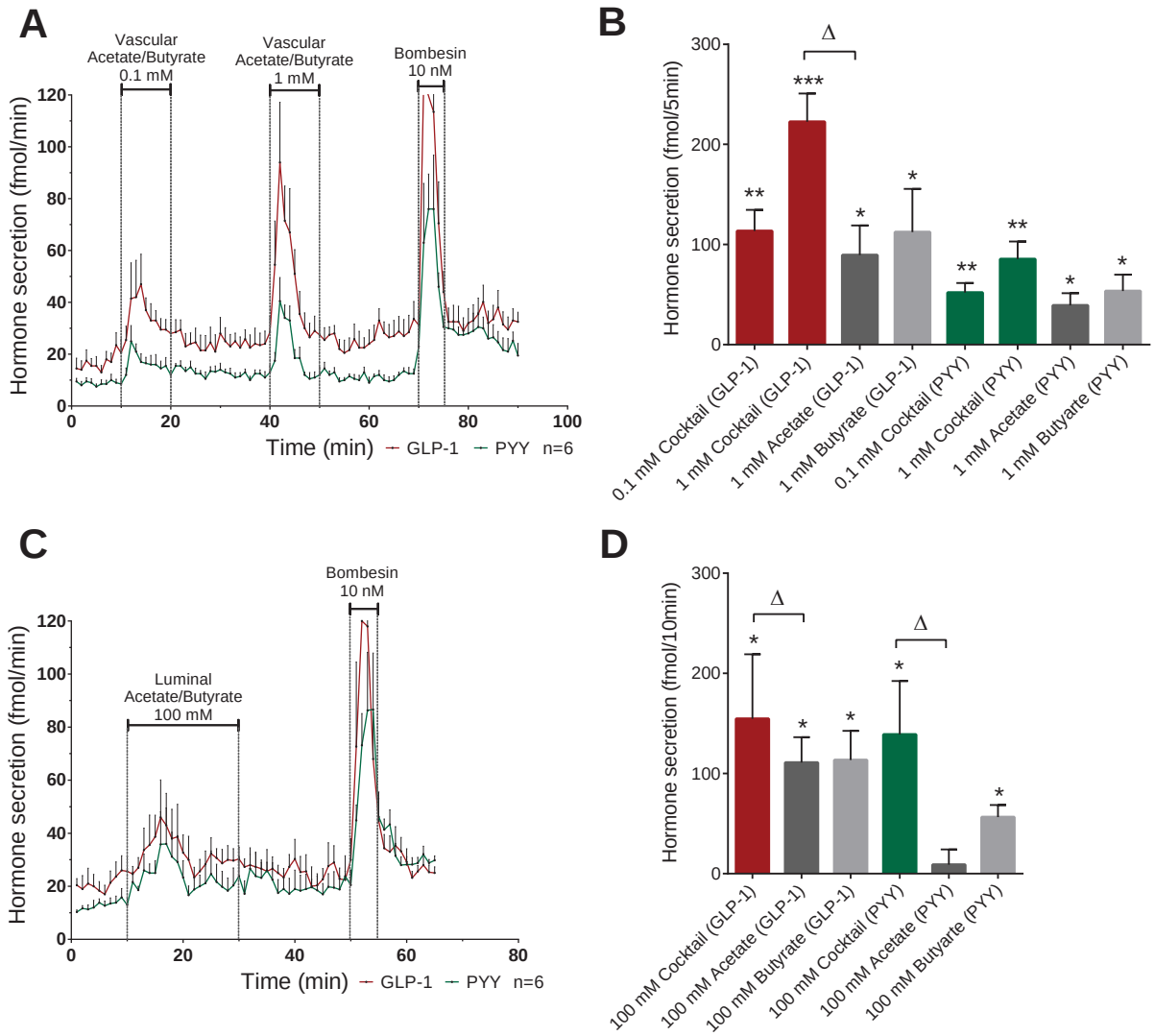
A
Vascular Acetate/Butyrate 0.1 mM
Vascular Acetate/Butyrate 1 mM
Bombesin 10 nM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=6
B
Hormone secretion (fmol/5min)
0.1 mM Cocktail (GLP-1)
1 mM Cocktail (GLP-1)
1 mM Acetate (GLP-1)
1 mM Butyrate (GLP-1)
0.1 mM Cocktail (PYY)
1 mM Cocktail (PYY)
1 mM Acetate (PYY)
1 mM Butyarte (PYY)
C
Luminal Acetate/Butyrate 100 mM
Bombesin 10 nM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=6
D
Hormone secretion (fmol/10min)
100 mM Cocktail (GLP-1)
100 mM Acetate (GLP-1)
100 mM Butyrate (GLP-1)
100 mM Cocktail (PYY)
100 mM Acetate (PYY)
100 mM Butyarte (PYY)

Figure 4

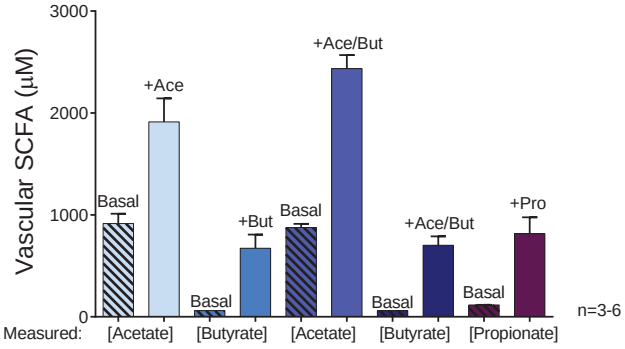
Vascular SCFA (μM)
3000
2000
1000
0
Basal
+Ace
Basal
+But
Basal
+Ace/But
Basal
+Ace/But
Basal
+Pro
n=3-6
Measured:
[Acetate]
[Butyrate]
[Acetate]
[Butyrate]
[Propionate]

Figure 5

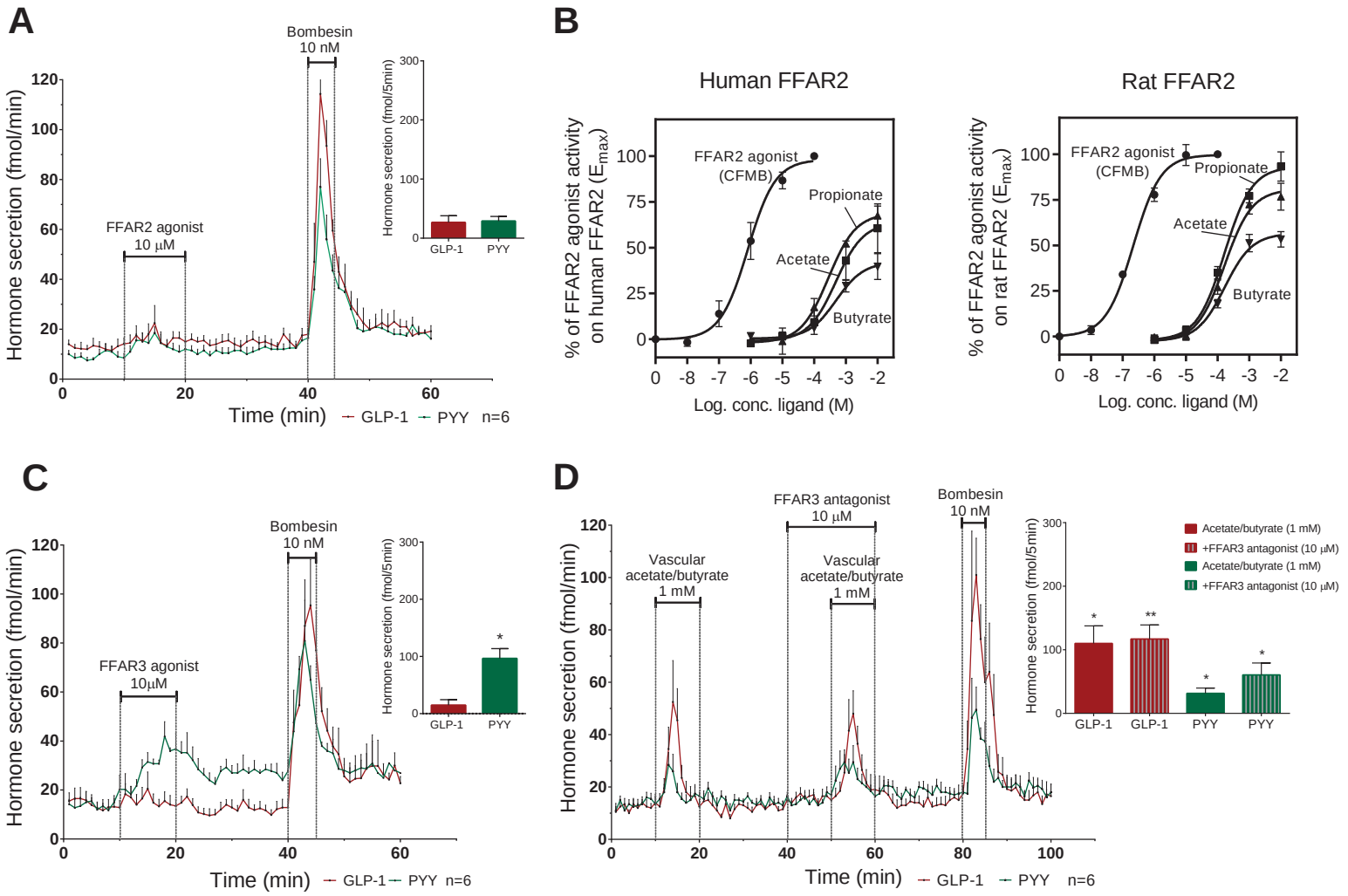
A
Bombesin
10 nM
FFAR2 agonist
10 μM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=6
Hormone secretion (fmol/5min)
B
Human FFAR2
Rat FFAR2
% of FFAR2 agonist activity on human FFAR2 (Emax)
% of FFAR2 agonist activity on rat FFAR2 (Emax)
FFAR2 agonist (CFMB)
Propionate
Acetate
Butyrate
Log. conc. ligand (M)
C
FFAR3 agonist
10μM
D
FFAR3 antagonist
10 μM
Vascular acetate/butyrate
1 mM
Acetate/butyrate (1 mM)
+FFAR3 antagonist (10 μM)

Figure 6

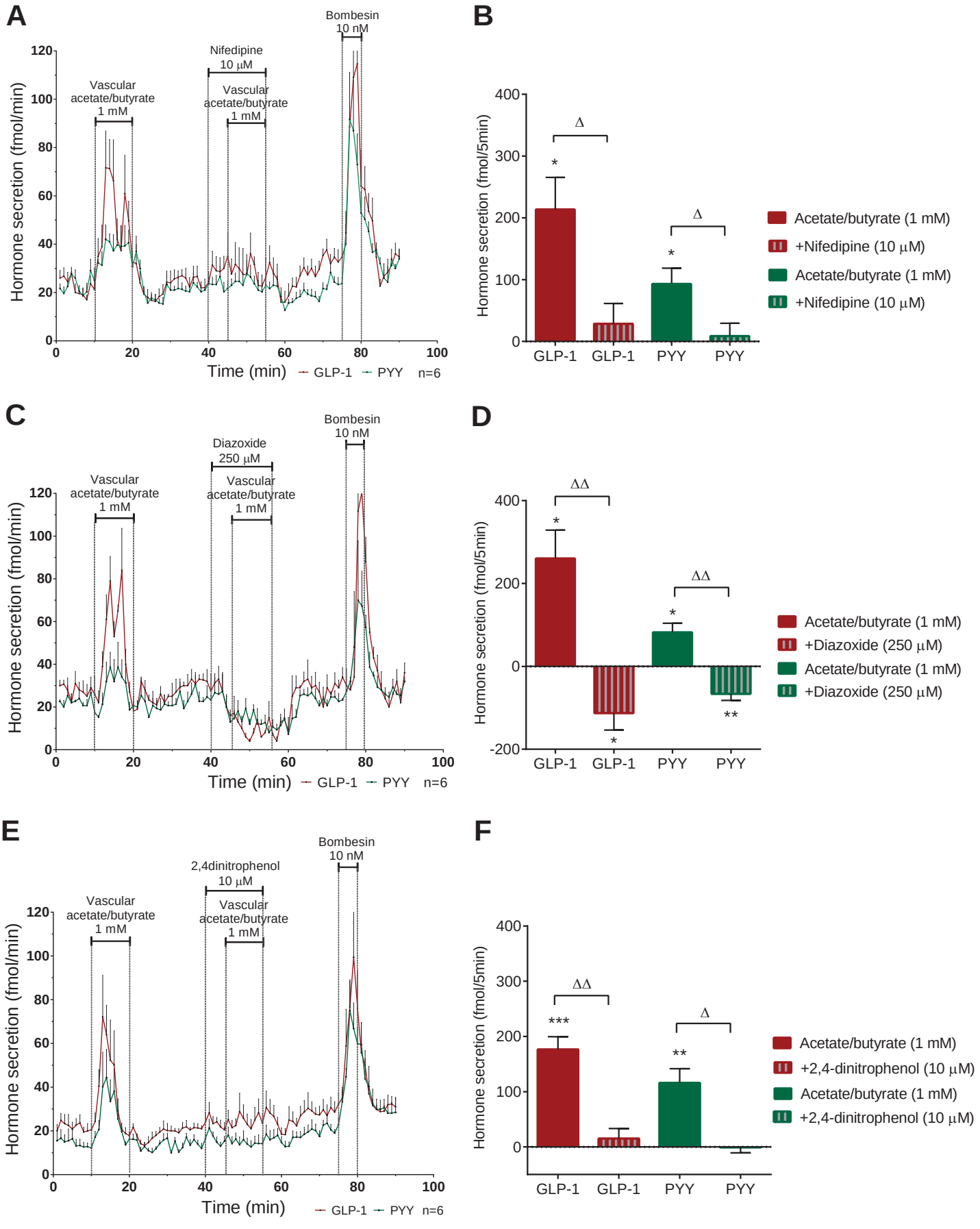
A
Bombesin
10 nM
Nifedipine
10 μM
Vascular
acetate/butyrate
1 mM
Vascular
acetate/butyrate
1 mM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=6
B
Hormone secretion (fmol/5min)
Δ
*
Δ
*
GLP-1
GLP-1
PYY
PYY
Acetate/butyrate (1 mM)
+Nifedipine (10 μM)
Acetate/butyrate (1 mM)
+Nifedipine (10 μM)
C
Bombesin
10 nM
Diazoxide
250 μM
Vascular
acetate/butyrate
1 mM
Vascular
acetate/butyrate
1 mM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=6
D
Hormone secretion (fmol/5min)
ΔΔ
*
ΔΔ
*
*
**
GLP-1
GLP-1
PYY
PYY
Acetate/butyrate (1 mM)
+Diazoxide (250 μM)
Acetate/butyrate (1 mM)
+Diazoxide (250 μM)
E
Bombesin
10 nM
2,4dinitrophenol
10 μM
Vascular
acetate/butyrate
1 mM
Vascular
acetate/butyrate
1 mM
Hormone secretion (fmol/min)
Time (min)
GLP-1
PYY
n=6
F
Hormone secretion (fmol/5min)
ΔΔ
***
Δ
**
GLP-1
GLP-1
PYY
PYY
Acetate/butyrate (1 mM)
+2,4-dinitrophenol (10 μM)
Acetate/butyrate (1 mM)
+2,4-dinitrophenol (10 μM)

Table 1

	Human FFAR2						Rat FFAR2					
	Log (EC_{50})	±SEM	EC_{50} (mM)	E_{max} of CFMB activity (%)	Fold change to EC_{50} of CFMB	n	Log (EC_{50})	±SEM	EC_{50} (mM)	E_{max} of CFMB activity (%)	Fold change to EC_{50} of CFMB	n
CFMB	-6.09	0.11	0.0008	100	1	4	-6.66	0.07	0.0002	100	1	3
Acetate	-3.32	0.30	0.48	64	589	4	-3.78	0.12	0.17	81	759	3
Propionate	-3.55	0.15	0.28	69	339	4	-3.78	0.10	0.17	93	759	3
Butyrate	-3.30	0.18	0.50	42	617	4	-3.77	0.13	0.17	57	776	3